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Meizothrombin Formation during Factor Xa-catalyzed Prothrombin Activation

FORMATION IN A PURIFIED SYSTEM AND IN PLASMA*

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Meizothrombin and thrombin formation were quantitated during factor Xa-catalyzed activation of human prothrombin in reaction systems containing purified proteins and in plasma. In the purified system considerable amounts of meizothrombin accumulated when prothrombin was activated by factor Xa (with or without accessory components) under initial steady state conditions. The ratio of the rates of meizothrombin and thrombin formation was not influenced by variation of the pH, temperature, or ionic strength of the reaction medium. When 2 μM prothrombin was activated by the complete prothrombinase complex (factor Xa, factor Va, Ca^{2+}, and phospholipid) 80–90% of the initially formed reaction product was meizothrombin. Lowering the prothrombin concentration from 2 to 0.03 μM caused a gradual decrease in the ratio of meizothrombin/thrombin formation from 5 to 0.6. When the phosphatidylserine content of the phospholipid vesicles was varied between 20 and 1 mol % and prothrombin activation was analyzed at 2 μM prothrombin the relative amount of meizothrombin formed decreased from 85 to 55%. With platelets, cephalin, or thromboplastin as procoagulant lipid, thrombin was the major reaction product and only 30–40% of the activation product was meizothrombin. We also analyzed complete time courses of prothrombin activation both with purified proteins and in plasma. In reaction systems with purified proteins substantial amounts of meizothrombin accumulated under a wide variety of experimental conditions. However, little or no meizothrombin was detected in plasma in which coagulation was initiated via the extrinsic pathway with thromboplastin or via the intrinsic pathway with kaolin plus phospholipid (cephalin, platelets, or phosphatidylserine-containing vesicles). Thus, thrombin was the only active prothrombin activation product that accumulated during *ex vivo* coagulation experiments in plasma.

The conversion of prothrombin into α-thrombin by coagulation factor Xa is one of the central reactions of blood coagulation (for recent reviews, see Refs. 1, 2). Thrombin formation is the result of cleavage of the Arg^{273}-Thr^{274} and Arg^{322}-Ile^{323} bonds in the prothrombin molecule, and depending upon the order of peptide bond cleavage two intermediary products (prethrombin 2 and meizothrombin)[1] may accumulate during prothrombin activation. It is now well established that both prethrombin 2 (3–5) and meizothrombin (2, 5–8) can indeed be formed during factor Xa-catalyzed prothrombin activation, which means that two enzymatically active reaction products, *i.e.* thrombin and meizothrombin may accumulate when prothrombin is activated by factor Xa. Kinetic studies of prothrombin activation in reaction systems with purified bovine proteins under steady state (initial rate) conditions have shown that meizothrombin is actually the major reaction product which accounts for 70–80% of the amidolytic activity generated (6, 7). Although both thrombin and meizothrombin have nearly identical activity toward the chromogenic substrate S2238, they exhibit different activities toward physiological thrombin substrates. Meizothrombin has been reported to have less than 5% of the activity of thrombin toward fibrinogen (6, 9–12), factor V (12), and platelets (12). Thombin and meizothrombin are equally well inhibited by the plasma protease inhibitor AT III[2] (13). Heparin does not promote the inhibition of meizothrombin by AT III whereas it greatly accelerates the inactivation of thrombin by this inhibitor (13). It has been reported (12), however, that meizothrombin is able to activate protein C with about 75% of the activity of thrombin. Based on the observation that meizothrombin lacks the procoagulant properties of α-thrombin whereas it exhibits considerable activity in protein C activation, it has been postulated that meizothrombin may have an anticoagulant function and that the generation of α-thrombin may predominantly serve procoagulant functions (12).

In view of a possible role of meizothrombin in the regulation of hemostatic plug formation, it is of interest to know whether different experimental conditions influence the relative rates at which thrombin and meizothrombin are produced during prothrombin activation. In the present study we investigated in a model system with purified human proteins the effect of

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[1] The nomenclature used for proteolytically derived products of prothrombin is that recommended by the International Committee on Thrombosis and Haemostasis (Jackson, C. M. (1977) *Thromb. Haemostasis* **38,** 567–577).

[2] The abbreviations used are: AT III, antithrombin III; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; $α_2$-M, $α_2$-macroglobulin; BSA, bovine serum albumin; DOPC, 1,2-dioleoyl-*sn*-glycerophosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycerophosphoserine; DOPE, 1,2-dioleoyl-*sn*-glycerophosphoethanolamine; egg-PC, egg yolk phosphatidylcholine; brain-PS, bovine brain phosphatidylserine; I2581, *N*-dansyl-(*p*-guanidino)-phenylalanine-piperidide hydrochloride; S2238, D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide; PPACK, phenylalanyl-prolyl-arginine chloromethyl ketone; *p*-NPGB, *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride; RVV-X, purified activator from Russell's viper venom.

different reaction conditions (*i.e.* prothrombinase composition, protein concentrations, phospholipid composition and concentration, and pH, temperature, and ionic strength of the reaction medium) on meizothrombin and α-thrombin formation. Furthermore, we have analyzed prothrombin activation in plasma in an attempt to establish whether meizothrombin accumulates during coagulation of plasma.

EXPERIMENTAL PROCEDURES

Materials

Ovalbumin (grade V), benzamidine-HCl, Russell's viper venom, *Echis carinatus* venom, soybean trypsin inhibitor (Type IS), rabbit brain cephalin, egg yolk PC, DOPC, DOPE, bovine brain PS, and the calcium-ionophore A23187 were purchased from Sigma. Kaolin (light) was from BDH. Cholesterol (99% pure) was obtained from Merck. Horse tendon collagen (Type I) was from Hormon Chemie, Munich, Germany and I2581, S2337, and S2238 were obtained from AB Kabi Diagnostica, Sweden. PPACK was purchased from Calbiochem, La Jolla, CA. *p*-NPGB was obtained from Nutritional Biochemicals. Reptilase reagent was from Boehringer, Mannheim, Germany. Agarose (Indubiose A37-HAA) was from IBF Biotechnics, France. Crude porcine intestinal mucosal heparin (175 USP/mg) was from Organon, The Netherlands. Swine anti-rabbit IgG horseradish peroxidase-conjugated antibodies were obtained from Nordic, Tilburg, The Netherlands. 3.3′-Diaminobenzidinetetrahydrochloride was from Fluka AG, Switzerland. All other reagents were of the highest grade commercially available.

Methods

Proteins—The proteins used in this study were purified from fresh frozen human plasma. Prothrombin and factor X were purified according the method of diScipio *et al.* (14). AT III was isolated as described by Thaler and Schmer (15). Factor Xa was obtained from purified factor X after activation with RVV-X and isolation of the factor Xa from the activation mixture by affinity chromatography on soybean trypsin inhibitor-Sepharose as described by Bock *et al.* (16). RVV-X was purified from the crude venom of Russell's viper according the method of Schiffmann *et al.* (17). α-Thrombin was prepared by activation of prothrombin (20 mg) at 37 °C in 10 ml of buffer containing 50 mM Tris, pH 7.9, 100 mM NaCl, 2 mM $CaCl_2$, 50 μM phospholipid vesicles (brain-PS/egg-PC, 20/80; mol/mol), 2.5 nM factor Xa, 4 nM factor Va, and 20 μM I-2581. The reversible thrombin inhibitor I2581 was present to prevent autocatalytic degradation of α-thrombin. Prothrombin activation was complete within 10 min after which the α-thrombin was purified from the reaction mixture as described by Pletcher and Nelsestuen (18). Meizothrombin, meizothrombin-des-fragment 1, and thrombin used to set up the assays were freshly prepared before each experiment as follows. Meizothrombin was obtained by activation of 1 μM prothrombin with *E. carinatus* venom in the presence of the reversible thrombin inhibitor I2581. Prothrombin was activated at 37 °C in a total volume of 200 μl of buffer containing 50 mM Tris, pH 7.9, 175 mM NaCl, 5 mM $CaCl_2$, 20 μM I2581, and 20 μg/ml *E. carinatus* venom. Under these conditions all prothrombin was converted into meizothrombin within 1 min. Due to the presence of the thrombin inhibitor I2581, there was negligible formation of meizothrombin-des-fragment 1. The reaction was stopped after 1 min by diluting the reaction mixture 10-fold with ice-cold buffer. Meizothrombin-des-fragment 1 was prepared by activating 4 μM prothrombin at 37 °C in 200 μl of buffer containing 50 mM Tris, pH 7.9, 175 mM NaCl, 5 mM $CaCl_2$, and 40 μg/ml *E. carinatus* venom. After 2 min all prothrombin had been converted to meizothrombin-des-fragment 1 and the reaction was stopped by diluting the reaction mixture 40-fold into ice-cold buffer. Thrombin was obtained from the same reaction mixture by allowing autocatalysis to proceed for 1 h at 37 °C during which all meizothrombin-des-fragment 1 was quantitatively converted to thrombin (*i.e.* thrombin lacking residues 1–13 of the A-chain). The meizothrombin and meizothrombin-des-fragment 1 thus prepared were used within 1 h during which time period no further autocatalysis occurred. When meizothrombin and meizothrombin-des-fragment 1 were prepared for gel electrophoretic experiments the reaction samples were quenched with gel sample buffer.

Factor V was purified essentially as described by Dahlbäck and colleagues (19, 20) with minor modifications. To fresh frozen plasma the irreversible thrombin inhibitor PPACK was added to a final concentration of 2 μM. All buffer solutions used in the isolation procedure contained 250 nM PPACK. The final purification step (chromatography on AcA22) was performed with a buffer solution that did not contain PPACK. The presence of PPACK was necessary to obtain reproducible preparations of homogeneous and single chain factor V (>95%). Factor Va was prepared by activation of factor V (0.3 mg/ml) at 37 °C for 30 min with 30 nM thrombin in 50 mM Tris, pH 7.9, 175 mM NaCl, 20% (v/v) glycerol, and 5 mM $CaCl_2$. After the activation was complete, 30 nM PPACK was added to inhibit the thrombin present in the activation mixture. The small amount of remaining PPACK did not interfere with the reactions carried out with the factor Va preparations. Factor Va was subsequently diluted 1/10 in 10 mM Hepes, pH 7.5 at room temperature, 136 mM NaCl, 5 mM $CaCl_2$, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, and 5 mg/ml BSA and stored at −80 °C. Factor Va was stable for several months at −80 °C and lost about 10% of its activity when kept on ice for 6 h.

Protein preparations were homogeneous and >95% pure as judged by polyacrylamide gel electrophoresis in the presence of SDS according to Laemmli (21). Protein concentrations were routinely determined according to Lowry *et al.* (22) using BSA as a standard. Molar concentrations of thrombin (23) and factor Xa (24) were determined by active site titration with *p*-NPGB. Molar concentrations of meizothrombin and meizothrombin-des-fragment 1 were determined with the chromogenic substrate S2238 using a calibration curve made with known amounts of thrombin as a standard. Prothrombin concentrations were determined after complete activation of prothrombin with *E. carinatus* venom after which the amidolytic activity obtained was used to calculate the original amount of prothrombin present. Concentrations of factor Va were determined kinetically as described for bovine factor Va by Lindhout *et al.* (25).

Lipid Preparations—DOPS was prepared from DOPC by enzymatic synthesis according to Comfurius and Zwaal (26). Phospholipid vesicles were prepared as described earlier (4). Human brain thromboplastin was purified according to a modification of the method of Owren and Aas (27) as described by van Dam-Mieras *et al.* (28) and was stored at −80 °C in Tris-buffered saline (10 mM Tris, pH 7.4, 150 mM NaCl). Rabbit brain cephalin was reconstituted in Tris-buffered saline according to the manufacturer's instructions.

Defibrinated Plasma Preparations—Normal human plasma, obtained as described in van Dam-Mieras *et al.* (28), was defibrinated prior to use by incubation of 1 ml of plasma with 25 μl of reptilase reagent for 20 min at 37 °C. The plasma was placed on ice for 10 min, and the precipitated fibrin was subsequently removed by centrifugation. The defibrinated plasma thus obtained contains normal levels of coagulation factors (29). Defibrinated plasma was used to facilitate sampling from plasma-containing reaction mixtures in which otherwise clotting would have occurred. As judged by control experiments with purified proteins, the presence of reptilase did neither interfere with the assays of prothrombin activation products nor with the time courses of prothrombin activation.

Platelet Preparations—Washed human platelets were obtained from healthy consenting donors by venipuncture and isolation of the platelets as described before (30). In the final step of the isolation procedure, the platelets were resuspended at a final concentration of 1×10^9/ml in 10 mM Hepes, pH 7.5 at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, and 0.5% BSA. Platelet concentrations were determined with a Coulter counter.

Gel Electrophoretic Procedures—Gel electrophoresis of prothrombin activation products was carried out on 10% polyacrylamide slab gels (5% stacking gel) in the presence of SDS according to Laemmli (21) in a Protean II cel from Bio-Rad. Electrophoretic transfer to nitrocellulose sheets was carried out as described by Towbin *et al.* (31). Transfer was routinely performed overnight at 10 °C with 125-mA current in a Protean II blot module from Bio-Rad. Visualization of amidolytically active prothrombin activation products as well as immunologic detection of prothrombin and its activation products on the nitrocellulose sheets were performed as described earlier (8).

Quantitative Analysis of Prothrombin Activation—Rates of prothrombin activation in the purified system were determined under initial rate conditions as described earlier (4). Quantitation of thrombin and meizothrombin[3] was performed as described before (6) by

[3] Our amidolytic assays do not discriminate between meizothrombin and meizothrombin-des-fragment 1 since the activities of both enzymes toward S2238 are the same and they exhibit the same kinetics of inhibition by AT III plus heparin. In those cases where meizothrombin formation is quantitated it actually represents the sum of meizothrombin and meizothrombin-des-fragment 1 present in the reaction mixture.

determination of the total amidolytic activity toward S2238 which measures both enzymatically active prothrombin activation products, thrombin and meizothrombin (assay 1), and by determination of the activity remaining after 1 min of incubation with 5 nM AT III in the presence of 20 μg/ml heparin which only measures meizothrombin (assay 2). From the amounts of activated prothrombin (assay 1) and of meizothrombin present (assay 2), the initial rates of prothrombin activation and meizothrombin formation were calculated. The rate of thrombin formation was subsequently calculated from the difference between the overall rate of prothrombin activation and the rate of meizothrombin formation.

When prothrombin activation is monitored in plasma, α_2-M-inhibited prothrombin activation products contribute to the amidolytic activity (29, 32), measured in both assays, and this precludes direct quantitation of thrombin and meizothrombin. Thus, an additional third amidolytic assay was performed in order to quantitate α_2-M-inhibited prothrombin activation products. In this latter assay an aliquot from the reaction mixture was transferred to a cuvette containing 25 nM AT III and 20 μg/ml heparin. Under these conditions thrombin and meizothrombin were completely inhibited, whereas the activity of α_2-M-inhibited thrombin species remained unaffected (see also "Results").

RESULTS[4]

In this paper we have used two different approaches to analyze the kinetics of time courses of prothrombin activation. In the model system (first part of this paper), we have determined the effect of the reaction conditions on initial rates of meizothrombin and thrombin formation. The experimental conditions were chosen such that prothrombin was always present in excess and that rates of meizothrombin and thrombin formation were constant in time indicating that such low amounts of reaction intermediates (*e.g.* meizothrombin) were formed that their rate of production greatly exceeded the rate by which they were converted into thrombin. In our experimental setup, prothrombin activation was started by the addition of prothrombin to reaction mixtures in which factor Xa was preincubated for 5 min at 37 °C with accessory components (factor Va, Ca^{2+}, and/or phospholipid). Under these conditions there was no delay in the onset of prothrombin activation. Rates of meizothrombin and thrombin formation were calculated from the amounts of product generated in the initial phase of the reaction (activation times <5 min) over a time interval in which less than 4% of the prothrombin present was converted into enzymatically active products (thrombin, meizothrombin). In the plasma system (second part of this paper), we have analyzed complete time courses of activation thus allowing further conversion of accumulated reaction intermediates.

It should be emphasized that we have only quantitated the generation of enzymatically active prothrombin activation products (thrombin and meizothrombin[3]) and that we did not consider the formation of prethrombin 2. This intermediate is only formed at appreciable amounts when prothrombin is activated by factor Xa in the absence of factor Va (4, 5, 33).

The Effect of the Composition of the Prothrombin-activating Complex on Initial Rates of Meizothrombin and Thrombin Formation—Table I shows the effect of variation of the composition of the prothrombin-activating complex on the overall rate of prothrombin activation and on the relative amounts of thrombin and meizothrombin that are formed in the initial phase of the reaction. This experiment was carried out at 2 μM prothrombin, which approximates the plasma prothrombin concentration. In agreement with the literature (*cf.* Refs. 1, 2) the accessory components, factor Va and phospholipid, greatly stimulated the overall rate of prothrombin activation. Analysis of the amidolytically active products generated indicated that with all compositions of the prothrombinase complex substantial amounts of meizothrombin were formed. With a prothrombin-activating complex consisting of factor Xa, factor Va, and $CaCl_2$, the rate of meizothrombin accumulation was some 4-fold lower than the rate of thrombin formation. In all other cases meizothrombin was formed in excess of thrombin and accounted for about 80% of the enzymatically active product generated under initial steady state rate conditions. These results are in agreement with data that we obtained earlier with bovine coagulation factors (2, 6).

TABLE I

Initial rates of thrombin and meizothrombin formation in the absence and presence of accessory components

Initial rates of prothrombin activation were determined as follows. Factor Xa was preincubated at 37 °C in 335 μl of buffer containing 10 mM Hepes, pH 7.5 at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA in the absence or presence of $CaCl_2$, factor Va, and phospholipid vesicles (DOPS/DOPC, 80/20; mol/mol). After 5 min, the reaction was started with the addition of 15 μl of prothrombin in the same buffer. The final concentrations reached in the reaction mixture were 10 mM Hepes, pH 7.5 at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 2 μM prothrombin, and amounts of $CaCl_2$, factor Xa, factor Va, and phospholipid vesicles (PL) as indicated in the table. In the case where $CaCl_2$ was absent the reaction was carried out in the presence of 5 mM EDTA. The initial rates of thrombin and meizothrombin (plus meizothrombin-des-fragment 1) formation were determined as described earlier (4, 6). For further experimental details see also under "Experimental Procedures."

Activator	v (mol/min/mol Xa)		% meizo
	Thrombin formation	Meizothrombin formation	
Xa (25 nM)	0.0011	0.0034	76
Xa (40 nM), $CaCl_2$ (2 mM)	0.0046	0.0110	71
Xa (4 nM), $CaCl_2$ (2 mM), PL (100 μM)	0.0718	0.4235	86
Xa (4 nM), $CaCl_2$ (2 mM), Va (60 nM)	5.69	1.46	20
Xa (4 pM), $CaCl_2$ (2 mM), Va (4 nM), PL (100 μM)	619	5016	89

The experiment presented in Table I was performed at optimal concentrations of accessory components. The relative amounts of meizothrombin and thrombin formed with each composition of prothrombinase complex were, however, not influenced by variation of the amounts of factor Xa or factor Va present nor were they affected by changing the concentrations of $CaCl_2$ (0.5–10 mM) or phospholipid (5–200 μM in the absence of factor Va and 0.5–200 μM in the presence of factor Va; data not shown). The phospholipid concentration ranges were chosen such that the contribution of the reaction in free solution remained negligible, and product generation was the result of lipid-dependent prothrombin activation.

The Effect of Variation of pH, Temperature, and Ionic Strength on Thrombin and Meizothrombin Formation during Prothrombin Activation—Fig. 1 (Miniprint) shows the effect of variation of the pH, ionic strength, and temperature on the overall rate of prothrombin activation, *i.e.* meizothrombin plus thrombin formation (Fig. 1, *A–C*) and on the amount of meizothrombin formed (expressed as the percent of the total amount of active product, *i.e.* thrombin plus meizothrombin, Fig. 1, *D–F*) in the initial phase of prothrombin activation by the complete prothrombinase complex (factor Xa, $CaCl_2$, factor Va, and phospholipid). Although the overall rate of prothrombin activation was strongly dependent on the pH, ionic

[4] Portions of this paper (Figs. 1, 2*A*, 3*A*, 7, and 8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

strength, and temperature of the reaction medium, variation of these parameters did not cause significant changes in the relative amounts of meizothrombin and thrombin that were formed. Also, variation of pH, ionic strength, and temperature hardly influenced the ratio of meizothrombin over thrombin formed when prothrombin was activated by prothrombinase complexes of different composition (data not shown).

It appears, therefore, that the data given in Table I are representative for a wide variety of experimental conditions and that the relative contributions of meizothrombin and thrombin to the overall rate of prothrombin activation are not influenced by changes in pH, temperature, or ionic strength of the reaction medium.

Effect of Prothrombin Concentration—The initial rates of thrombin and of meizothrombin formation were determined at different prothrombin concentrations, and the amount of meizothrombin formed was expressed as percentage of the total amount of active product (thrombin plus meizothrombin) generated during the initial steady state of prothrombin activation. The dependence of the overall rate of prothrombin activation as a function of prothrombin concentration is given in Fig. 2*A* (Miniprint). Fig. 2*B* shows that when prothrombin was activated by the complete prothrombinase complex (factor Xa, factor Va, $CaCl_2$, and phospholipid) the contribution of meizothrombin to product generation during prothrombin activation was dependent upon the prothrombin concentration and increased from 40% at 0.03 μM prothrombin to about 85% at 2 μM prothrombin (Fig. 2*B*). However, in the absence of factor Va the relative amounts of meizothrombin formed were independent of the prothrombin concentration, and 80–90% of the active product generated was meizothrombin irrespective of the amount of prothrombin present in the activation mixture (Fig. 2*B*) and of the large variation of the overall rate of prothrombin activation (Fig. 2*A*).

Lipid Membrane Composition—It is well known that the lipid composition of procoagulant membranes may strongly influence their ability to accelerate coagulation factor activation. Procoagulant membranes require the presence of negatively charged phospholipids such as phosphatidylserine, the major anionic phospholipid in blood cell membranes. In the experiment presented in Fig. 3, we have measured the influence of variation of the amount of phosphatidylserine, present in procoagulant membranes, on the overall rate of prothrombin activation and on the rates of thrombin and meizothrombin formation. The effect of the variation of the mole fraction of phosphatidylserine on the overall rate of prothrombin activation is shown in Fig. 3*A* (Miniprint). The different phosphatidylserine requirements observed for prothrombin activation with and without factor Va is in agreement with an earlier report on bovine prothrombin activation (34). Fig. 3*B* shows that the mole fraction of phosphatidylserine present in phospholipid vesicles also affected the relative amounts of meizothrombin and thrombin formed irrespective of whether factor Va was present or not. When the phosphatidylserine content of the vesicles was increased from 1 to 40 mol % the contribution of meizothrombin to the overall rate of prothrombin activation increased from some 55% to approximately 85%.

Table II summarizes initial rates of thrombin and meizothrombin formation during prothrombin activation by factor Xa, factor Va, and $CaCl_2$ in the presence of various procoagulant membranes. The data presented in this table indicate that the relative amounts of thrombin and meizothrombin formed were strongly dependent upon the choice of membrane. At 2 μM prothrombin, DOPS/DOPC (10:90, mol/mol) vesicles yielded some 90% meizothrombin whereas in the presence of lipids from natural sources such as human brain thromboplastin, rabbit brain cephalin, or platelets much less meizothrombin was generated. Artificial vesicles that mimic the composition of the platelet membrane (a mixture of DOPS, DOPI, DOPC, DOPE, sphingomyelin, and cholesterol at a molar ratio of 10:3:37:30:20:50) also showed reduced meizothrombin formation.

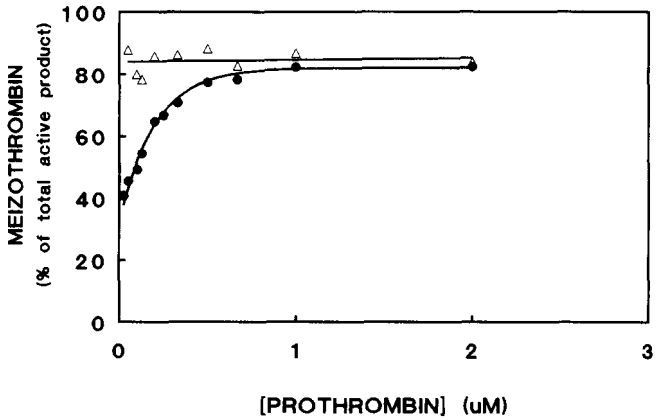

FIG. 2B. **Meizothrombin formation as a function of the prothrombin concentration.** The initial rate of prothrombin activation and of meizothrombin formation was determined at varying prothrombin concentrations in the absence or presence of factor Va. The dependence of the rate of prothrombin activation on prothrombin concentration is given in *A*. Meizothrombin formation (*B*) is expressed as percentage of the total amount of enzymatically active prothrombin activation product (thrombin plus meizothrombin) formed at each prothrombin concentration. Further details are given in the legend to Fig. 1 and under "Experimental Procedures." The final concentrations of reactants were: 50 mM Hepes, pH 7.5 at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 2 mM $CaCl_2$, 50 μM DOPS/DOPC vesicles (10:90; mol/mol), prothrombin concentrations as indicated in the figure and 4 nM factor Xa without factor Va (△) or 3 pM factor Xa and 5 nM factor Va (●).

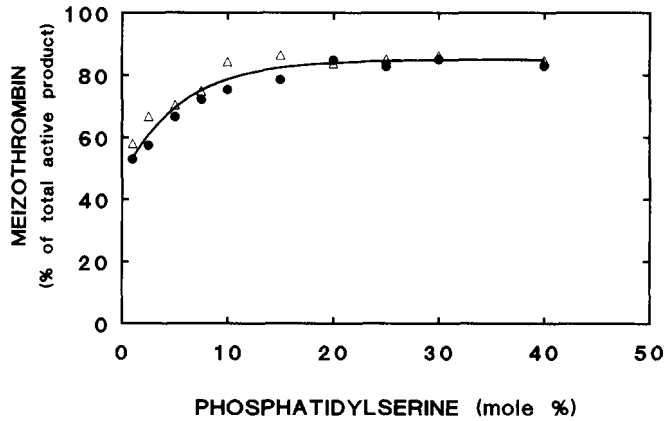

FIG. 3B. **Effect of the membrane composition on meizothrombin formation.** The initial rate of prothrombin activation and of meizothrombin formation were determined on DOPS/DOPC vesicles of varying composition both in the absence and presence of factor Va. The dependence of the overall rate of prothrombin activation on membrane composition is given in *A*. Meizothrombin formation is expressed as percentage of the total amount of enzymatically active prothrombin activation product (thrombin plus meizothrombin) formed at each phospholipid composition. Further details are given in the legend to Fig. 1 and under "Experimental Procedures." The final concentrations of reactants were: 50 mM Hepes, pH 7.5 at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 50 μM DOPS/DOPC vesicles with varying mol % DOPS as indicated in the figure, 2 μM prothrombin, 2 mM $CaCl_2$, and 3.5 nM factor Xa and no factor Va (△) or 3.5 pM factor Xa and 5 nM factor Va (●).

TABLE II

Initial rates of thrombin and meizothrombin formation with various procoagulant membranes

The initial rates of thrombin and meizothrombin (plus meizothrombin-des-fragment 1) formation by factor Xa, factor Va, and $CaCl_2$ in the presence of different procoagulant membranes were determined as described under the legend to Table I in a total volume of 350 μl of buffer at 37 °C. The final concentrations reached in the reaction mixture were 10 mM Hepes, pH 7.5 at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 2 μM prothrombin, 4 pM factor Xa, 7 nM factor Va, 2 mM $CaCl_2$, and 25 μM phospholipid vesicles, 10^8/ml sonicated or Ca^{2+}-ionophore-stimulated platelets, 25 μM cephalin, or thromboplastin. The concentration of thromboplastin given was based on a phosphate analysis of the lipid. The platelets were stimulated for 10 min with 4 μM ionophore before addition to the reaction mixture. For further experimental details see the legend to Table I and under "Experimental Procedures."

Membrane composition	*v* (mol/min/mol Xa)		% meizo
	Thrombin formation	Meizothrombin formation	
PS/PC (10/90)	1696	6407	79
PS/PI/PC/PE/Sph/Chol (molar ratio 10:3:37:30:20:50)	2768	5189	65
Stimulated platelets	4228	1094	21
Platelet sonicate	3527	3315	48
Thromboplastin	4446	1804	29
Cephalin	4310	2474	36

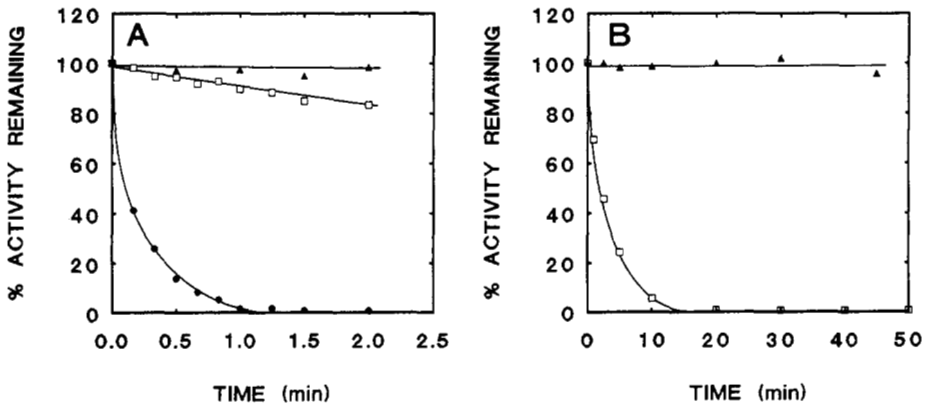

FIG. 4. **Inhibition of the amidolytic activity of thrombin, meizothrombin, and of the α_2-macroglobulin-thrombin complex by antithrombin III plus heparin.** Inhibition of thrombin, meizothrombin, or the α_2-macroglobulin-thrombin complex was determined at 37 °C in a series of 1-cm Sarstedt cuvettes with 950 μl of buffer containing 5 nM AT III + 20 μg/ml heparin (*panel A*) or 25 nM AT III + 20 μg/ml heparin (*panel B*). The final amounts of reactants present in the reaction mixtures were: *panel A*: ●, 1 nM thrombin;, □, 1 nM meizothrombin; ▲, 1 nM α_2-macroglobulin-thrombin complex, 5 nM AT III, 20 μg/ml heparin, 100 mM Tris, pH 7.9 at room temperature, 175 mM NaCl, 20 mM EDTA, and 0.5 mg/ml ovalbumin. *Panel B*: as in *panel A* except that 25 nM AT III was present, and the inhibition of thrombin was not determined. At the time intervals indicated, the remaining amidolytic activity was determined by addition of 50 μl of S2238 (200 μM final concentration) and was expressed as the percentage of the amidolytic activity measured in the absence of AT III and heparin.

Assays for the Detection and Quantitation of Amidolytically Active Prothrombin Activation Products in Plasma—When prothrombin activation is analyzed in plasma one should take into account that the active enzymes that are formed during coagulation of plasma are susceptible to inhibition by circulating proteinase inhibitors. The major plasma inhibitors of thrombin and presumably also of meizothrombin are AT III and α_2-M. Inhibition of thrombin and meizothrombin by AT III results in the complete disappearance of enzymatic activity, whereas α_2-M-inhibited thrombin and meizothrombin retain part of their activity toward small oligopeptide substrates. Thus, when prothrombin activation in plasma is monitored with the chromogenic substrate S2238 part of the amidolytic activity measured may originate from α_2-M-inhibited prothrombin activation products. The experiment shown in Fig. 4 illustrates that assay conditions can be found in which the chromogenic substrate S2238 can discriminate between the various amidolytically active products that are generated during prothrombin activation in plasma. Fig. 4*A* shows the inhibition of thrombin, meizothrombin, and the α_2-M-thrombin complex by 5 nM AT III in the presence of 20 μg/ml heparin. Under these conditions thrombin was completely inhibited within 1 min whereas there was a minor effect on the amidolytic activities of meizothrombin and the α_2-M-thrombin complex. When the amount of AT III present was raised to 25 nM all free enzyme (thrombin and meizothrombin) was inhibited within 20 min whereas the thrombin-α_2-M complex was not inhibited (Fig. 4*B*). These assays were not affected by plasma provided that the amount of plasma present was less than 0.3% (v/v). The addition of more plasma caused some underestimation of meizothrombin and as a consequence an overestimation of thrombin. Since the amounts of plasma required to follow prothrombin activation with S2238 were very low, it was possible to monitor product generation during the activation of prothrombin in plasma with a combination of three amidolytic assays, *i.e.* 1) determination of the total amidolytic activity which measures thrombin, meizothrombin, and the α_2-M complexes, 2) determination of the amidolytic activity after incubating a plasma sample for 1 min with 5 nM AT III plus 20 μg/ml heparin which measures meizothrombin plus the α_2-M complexes, and 3) determination of the amidolytic activity after incubating a

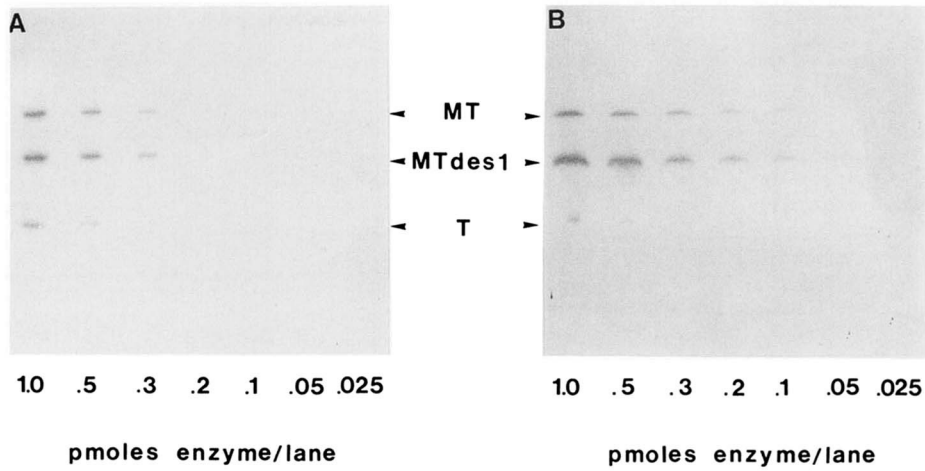

FIG. 5. **Visualization of amidolytically active prothrombin activation products after SDS-gel electrophoresis.** Amounts of meizothrombin, meizothrombin-des-fragment 1, and thrombin indicated in the figure were subjected to gel electrophoresis on 10% slabgels (5% stacking gel) according to Laemmli (21) in the presence of SDS with and without 2 μl of plasma present in each sample. The amidolytically active products were visualized after electrophoresis and transblotting to nitrocellulose as described earlier (8). *Panel A*, samples run in the absence of plasma. *Panel B*, samples with 2 μl of plasma present on each gel lane. Further experimental details are given under "Experimental Procedures."

plasma sample for 30 min with 25 nM AT III plus 20 μg/ml heparin which only measures the α_2-M-inhibited prothrombin activation products.

In a recent paper (8), we have shown that amidolytically active prothrombin activation products can be visualized after gel electrophoresis in the presence of SDS and transblotting to nitrocellulose. This technique could also be used to identify prothrombin activation products in plasma. Fig. 5*A* shows the gel electrophoretic analysis of samples containing various amounts of an equimolar mixture of meizothrombin, meizothrombin-des-fragment 1, and thrombin which indicated that rather low concentrations of enzymatically active prothrombin activation products can be detected by the amidoblot procedure. When the same amounts of prothrombin activation products were electrophoresed in the presence of 2 μl of plasma (Fig. 5*B*) the sensitivity for thrombin remained approximately the same while there was an as yet unexplained increase in the sensitivity for meizothrombin and meizothrombin-des-fragment 1. Larger amounts of plasma could not be applied to the gel since in that case too much smearing and loss of the meizothrombin-des-fragment 1 band occurred (data not shown). This experiment illustrates that the detection limits for meizothrombin, meizothrombin-des-fragment 1, and thrombin on the amidoblot of plasma samples were about 50, 25, and 150 nM, respectively.

Generation of Prothrombin Activation Products during Coagulation Initiated Via the Extrinsic Pathway—Using the techniques described above, the activation of prothrombin was monitored in defibrinated normal human plasma upon addition of human brain thromboplastin (Fig. 6). After approximately 15–20 s an explosive burst of amidolytic activity occurred which was followed by a rapid decrease in activity. After about 3–4 min a final plateau was reached representing α_2-M-inhibited prothrombin activation products (Fig. 6*A*). The vast majority of the burst of the amidolytic activity appeared to be associated with thrombin and little or no meizothrombin could be detected.[5] The highest thrombin concentration reached was approximately 650 nM, whereas the highest meizothrombin values determined remained below 25 nM and were in fact within experimental error. These findings were corroborated by electrophoretic analysis of prothrombin activation products present in plasma samples taken at the same time intervals (Fig. 6, *B* and *C*). In the amidoblot only thrombin was detectable as a transient band and no other amidolytically active reaction products were visible (Fig. 6*B*). Immunologic staining with a polyvalent anti-human prothrombin antibody showed that all prothrombin disappeared during the time course of activation (Fig. 6*C*). A band with the electrophoretic mobility of fragment 1.2 could clearly be seen, and the (transient) thrombin band was faintly visible underneath this band. At later time points a band with the electrophoretic mobility of fragment 1 became visible as well as two bands with higher M_r than prothrombin (presumably representing thrombin-AT III complexes). This experiment also indicates that there is no significant accumulation of meizothrombin during the extrinsic activation of plasma.

Generation of Prothrombin Activation Products during Coagulation Initiated via the Intrinsic Pathway—Fig. 7 (Miniprint) shows the result of an experiment in which prothrombin activation was monitored in defibrinated normal human plasma in which coagulation occurred via the intrinsic pathway with kaolin as initiator and with rabbit brain cephalin (Fig. 7*A*) or activated platelets as procoagulant lipid surface (Fig. 7*B*). In the latter case 10 μg/ml collagen was present to ensure optimal expression of platelet procoagulant activity (35). The appearance of amidolytic activity was later than in plasma activated via the extrinsic pathway and the maximal thrombin concentrations reached were lower. However, with respect to the generation of the various prothrombin activation products the data obtained in plasma activated via the intrinsic and extrinsic pathways were similar (*cf.* Figs. 6 and 7). It was observed that both in intrinsic- and extrinsic-activated plasma thrombin was the major prothrombin activation product and that little or no meizothrombin was detectable during the activation of plasma prothrombin.

Comparison of Product Generation during Prothrombin Activation in the Model System and in Plasma—The data presented thus far indicate that during prothrombin activation in the model system using purified coagulation factors and phospholipid vesicles prepared from synthetic phospholipids considerable amounts of meizothrombin are formed whereas in the plasma milieu hardly any meizothrombin is detectable. There are three major differences between the experiments in the model system and in plasma. 1) In the model system we used phospholipid vesicles composed of DOPS and DOPC while the plasma experiments were performed in the presence of lipids from natural sources (thromboplastin, cephalin, or

[5] The conversion of S2238 in the plasma experiments is almost exclusively due to thrombin and/or meizothrombin. Simultaneous measurements with the chromogenic substrates S2302 and S2337 indicated that the amounts of kallikrein, factor XIIa, and factor Xa formed were insufficient to contribute to S2238 conversion. The plasma concentrations of factor IXa and factor XIa and their low activities toward S2238 exclude the possibility that these enzymes contribute to the conversion of S2238.

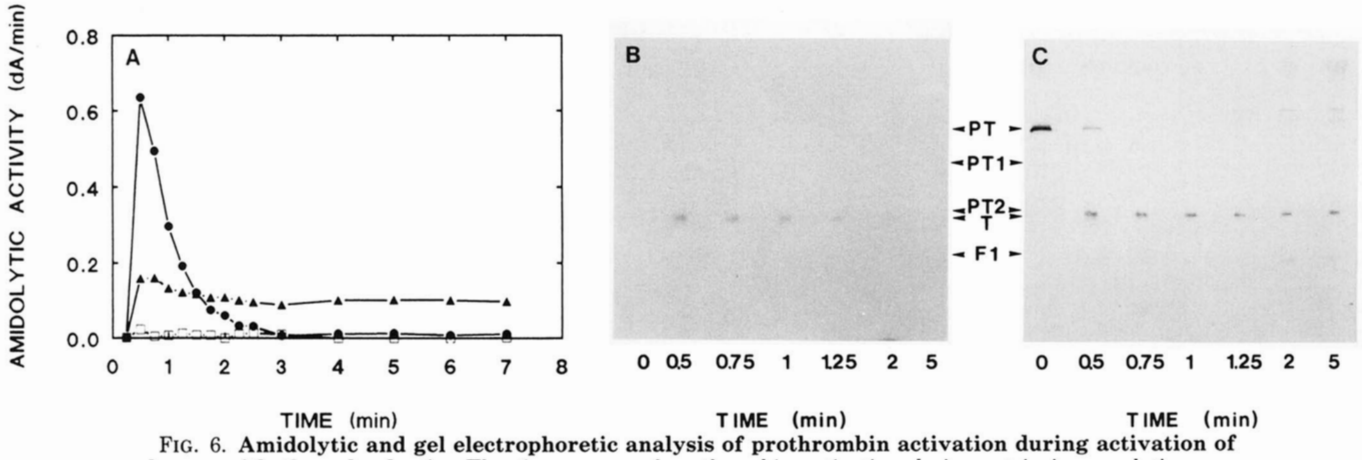

FIG. 6. **Amidolytic and gel electrophoretic analysis of prothrombin activation during activation of plasma with thromboplastin.** The time course of prothrombin activation during extrinsic coagulation was monitored as follows: 807 μl of defribrinated normal human plasma was preincubated for 5 min in a polystyrene tube at 37 °C with 100 μl of buffer, and 10-μl aliquots were withdrawn for analysis at time 0. Reaction was started with the addition of 100 μl of thromboplastin and 13 μl of 1 M $CaCl_2$, and at the times indicated in the figure 10-μl aliquots were withdrawn for quantitative analysis of the prothrombin activation products (*panel A*) and for gel electrophoretic analysis (*panels B* and *C*). The final concentrations of reactants reached were: 0.8 volume defibrinated plasma, 200 μM thromboplastin (by phosphate analysis), 20 mM Hepes, pH 7.5 at 37 °C, and 13 mM added $CaCl_2$. *Panel A*, the 10-μl aliquot was diluted 100-fold in ice-cold buffer (100 mM Tris, 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin) and the amidolytic activities (in arbitrary units) of thrombin (●, meizothrombin plus meizothrombin-des-fragment 1 (□), and α_2-macroglobulin-inhibited prothrombin activation products (▲) were determined as described under "Experimental Procedures." The highest value of thrombin reached equalled 0.63 μM. *Panel B*, the 10-μl aliquots were diluted 5-fold in SDS-electrophoresis buffer of which 20-μl samples were subjected to gel electrophoresis according to Laemmli (21) in the presence of SDS on 10% slabgels (5% stack). After electrophoresis the proteins were transblotted to nitrocellulose and the amidolytically active prothrombin activation products were visualized with S2238 as described earlier (8). *Panel C*, after the development and photography of the amidoblot the nitrocellulose sheet was washed and stained immunologically with a polyclonal rabbit anti-human prothrombin as first antibody and horseradish peroxidase-conjugated mouse anti-human IgG as second antibody. Further experimental details are given under "Experimental Procedures."

platelets) which tend to give rise to less meizothrombin (Table II). 2) Low concentrations of the factor Xa-factor Va complex were used in the model system whereas in plasma much higher amounts of this complex are generated. This might cause an increased rate of conversion of meizothrombin into thrombin. And finally, 3) the experiments performed in the model system were carried out in the absence of inhibitors while plasma contains AT III and α_2-M which might inhibit the thrombin and meizothrombin generated during prothrombin activation. We tested whether these differences could explain why meizothrombin does not accumulate in the plasma milieu.

The fact that meizothrombin did not accumulate in plasma (Figs. 6 and 7) was not simply due to the use of procoagulant lipids form natural sources (Table II). This is concluded from the fact that prothrombin activation during intrinsic coagulation in the presence of DOPS/DOPC vesicles (20/80; mol/mol) also did not result in the accumulation of detectable amounts of meizothrombin (data not shown).

The effect of high concentrations of prothrombinase on the time courses of product generation is shown in Fig. 8*A* (Miniprint). This experiment was performed in the model system at a concentration of factor Xa-factor Va complex which gave an initial rate of prothrombin activation approximately equal to the rate calculated from the burst of thrombin generation observed in the plasma experiment presented in Fig. 6. It can be seen that at such a high factor Xa and Va concentration in the model system meizothrombin was a transient intermediate that was rapidly formed and subsequently further converted into thrombin. However, it is also clear that in the initial phase ($t < 30$s) of prothrombin activation meizothrombin was formed in excess of thrombin and that for a rather long time period substantial amounts of meizothrombin were detectable. When the same experiment was carried out in the presence of 1.6 μM AT III and 2.5 μM α_2-M there was a considerable effect on the time courses of generation of thrombin and meizothrombin (Fig. 8*B*). For both products a short burst of formation was observed which was rapidly followed by product disappearance due to inhibition by the inhibitors present and in the case of meizothrombin presumably also by further processing into thrombin (*cf.* Fig. 8*A*). However, these experiments show that in the model system considerable amounts of meizothrombin still accumulated even when prothrombin was activated at high concentrations of factors Xa and Va and in the presence of the plasma protease inhibitors α_2-M and AT III.

DISCUSSION

The experiments presented in this paper were designed to gain insight in the potential significance of meizothrombin as a transient intermediate during the activation of human prothrombin. In the first part of this paper, we have described the effect of the experimental conditions on the initial rates of meizothrombin and thrombin formation in a reaction system containing purified human coagulation factors. Our data indicate that under almost all experimental conditions meizothrombin is the major enzymatically active reaction product formed in the initial phase of prothrombin activation. Thus, when prothrombin is activated by factor Xa alone or by factor Xa (± factor Va) in the presence of negatively charged phospholipid vesicles consisting of a mixture of DOPS and DOPC (20/80, mol/mol), meizothrombin accounts for 80–90% of the enzymatically active product generated under initial steady state rate conditions. The relative amounts of meizothrombin and thrombin that are formed are not influenced by variation of the pH, temperature, and ionic strength of the reaction medium nor are they affected by changes in the concentra-

tions of the accessory components, phospholipid, Ca^{2+} ions, and factor Va. Activation of prothrombin by factor Xa in the presence of Ca^{2+} and factor Va appears to be an exception, since under these conditions the rate of meizothrombin formation is about 4-fold lower than the rate of thrombin formation. The data obtained with prothrombinase complexes of different composition confirm earlier observations with bovine proteins (6). The accumulation of large amounts of meizothrombin during prothrombin activation by the complete prothrombinase complex in the purified system is also in agreement with the data of Krishnaswamy *et al.* (5, 7). Although these authors did not study meizothrombin formation in the absence of factor Va our data clearly indicate that also without factor Va the majority of initially formed enzymatically active reaction product is in fact meizothrombin. This is in agreement with the recent data of Carlisle *et al.* (33) who reported the generation of significant amounts of meizothrombin-des-fragment 1 in the initial phase of the activation of bovine prethrombin 1 by factor Xa in the absence of accessory components. It should be emphasized, however, that in the early phase of factor Xa-catalyzed prothrombin activation in the absence of factor Va, the non-active reaction intermediate prethrombin 2 is formed in excess of both meizothrombin and thrombin (4, 5, 33).

Parameters that do influence the ratio of the initial rates of meizothrombin and thrombin formation are the prothrombin concentration and the composition of the procoagulant membrane. At low concentrations of prothrombin, the majority of the enzymatically active product is thrombin while at high prothrombin concentrations more than 90% of the initially generated product is meizothrombin. This phenomenon is only observed when prothrombin is activated by the complete prothrombinase complex (factor Xa, factor Va, Ca^{2+} ions, and phospholipid). The composition of the procoagulant membrane also has a strong effect on the relative amounts of thrombin and meizothrombin that are formed. With the synthetic negatively charged PS/PC vesicles, that are regularly used in studies of prothrombin activation (20 mol % PS/80 mol % PC), meizothrombin appears to be formed in excess of thrombin. However, vesicles containing low amounts of PS tend to favor thrombin production. It was further observed that lipid preparations from physiological sources (cephalin, thromboplastin, or platelet membranes) yield much lower relative rates of meizothrombin formation. In such lipid suspensions thrombin is the predominant product in all phases of prothrombin activation. This may at least be partly caused by the fact that these lipid preparations contain relatively low amounts of PS (35–37).

In summary, reduced meizothrombin formation is observed (*a*) during prothrombin activation by the factor Xa-Va complex in the absence of phospholipids; (*b*) when prothrombin is activated at low prothrombin concentrations by the complete prothrombinase complex; and (*c*) when procoagulant membranes are used that contain low mole percentages of PS. We have as yet no mechanistic explanation for these phenomena. A detailed study of the effect of the different experimental conditions on the rate constants of cleavage of the Arg^{273}-Thr^{274} and Arg^{322}-Ile^{323} bonds in prothrombin may provide further information on this issue (*cf.* Ref. 33).

Surprisingly low amounts of meizothrombin did accumulate during prothrombin activation in plasma. Almost no meizothrombin was detectable during a time course of prothrombin activation in plasma in which the coagulation cascade was triggered either via the extrinsic or via the intrinsic pathway. The reduction of meizothrombin formation in plasma may be partially caused by the fact that lipid suspensions of physiological source (thromboplastin, cephalin, or platelets) tend to generate less meizothrombin. This can, however, not be the only explanation since the use of sonicated phospholipid vesicles composed of 20 mol % DOPS and 80 mol % DOPC in the plasma experiments did not result in a substantial accumulation of meizothrombin either.

Other obvious explanations exist for the fact that accumulation of meizothrombin in plasma will be far less than might intuitively be anticipated from extrapolation of the data obtained under steady state rate conditions in the purified system. It can be calculated from the burst of thrombin formation in plasma that under these conditions prothrombin is activated at a concentration of factor Xa-Va complexes of approximately 1 nM, which is much higher than the low factor Xa concentrations employed in the experiments in the model system (approximately 4 pM factor Xa). At such high enzyme concentrations meizothrombin can be readily converted into thrombin. Another reaction that might contribute to a decrease of meizothrombin accumulation is autocatalytic conversion of meizothrombin which results in thrombin lacking residues 1–13 of the A-chain (38). Autocatalysis may not be negligible at the high concentrations of thrombin (some 500 nM) generated in the plasma experiments. Finally, the presence of the plasma protease inhibitors AT III and α_2-M will also cause a reduction of the amount of meizothrombin formed in plasma milieu. Data obtained in the model system indicate that these inhibitors not only inhibit thrombin but may also be responsible for a rapid disappearance of meizothrombin. We feel, however, that these explanations do not fully account for the complete lack of meizothrombin accumulation in the plasma milieu as is illustrated by the experiment presented in Fig. 8. In this experiment, which was performed with purified proteins at high factor Xa and Va concentrations and in the presence of the plasma protease inhibitors AT III and α_2-M, considerable amounts of meizothrombin were formed. It seems, therefore, that a discrepancy exists between the plasma milieu and the model system concerning the accumulation of meizothrombin. Such a discrepancy can be caused by the fact that plasma prothrombin activation does not proceed via meizothrombin but for a unknown reason via prethrombin 2. It is also possible that plasma contains an as yet unidentified factor that either stimulates the conversion of meizothrombin into thrombin or that specifically and efficiently inhibits any meizothrombin that might accumulate in plasma. It is obvious that more experimental work is needed to explain the difference between the plasma and the model system and to obtain information about the possibilities that plasma factor(s) might interfere with product generation during the activation of plasma prothrombin.

The limited amounts of meizothrombin that are formed in our experiments carried out in the plasma system make it questionable whether meizothrombin formation has any physiological importance. In this respect it should be mentioned, however, that it has been recently reported that detectable amounts of meizothrombin and meizothrombin-des-fragment 1 accumulate as transient intermediates during prothrombin activation on endothelial cells (39). Considering the proposed function of meizothrombin in the anticoagulant pathway, *i.e.* the thrombomodulin-mediated activation of protein C on the endothelial cell (12), the formation of meizothrombin on the same cell surface may be of physiological significance. Since meizothrombin formation in the experiments in the endothelial cell system (39) was only assessed in a qualitative manner with purified proteins, it will be necessary to carefully quantitate meizothrombin formation and to study the effect of other plasma proteins in order to fully appreciate the impor-

tance of meizothrombin accumulation on the endothelial cell surface.

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Supplementary Material to Meizothrombin Formation During Factor Xa-Catalyzed Prothrombin Activation. FORMATION IN A PURIFIED SYSTEM AND IN PLASMA.

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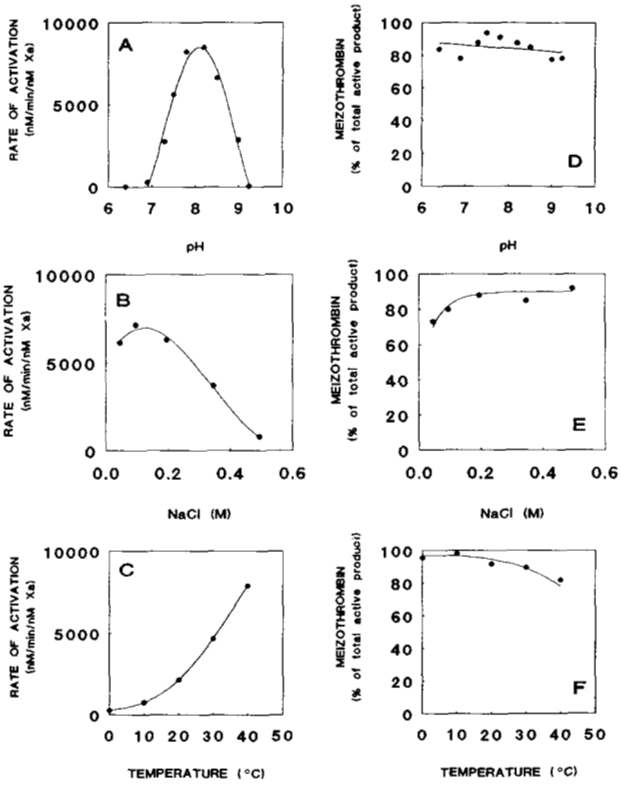

Figure 1. Meizothrombin formation as a function of pH, ionic strength and temperature.
Initial rates of prothrombin activation by the complete prothrombinase complex (Xa, Va, $CaCl_2$ and 20% DOPS/80% DOPC vesicles) were determined at varying pH, ionic strength or temperature in 350 µl total reaction volume as follows: factor Xa, factor Va and phospholipid vesicles were preincubated for 5 min in the presence of 2 mM $CaCl_2$ in 335 µl reaction buffer after which the reaction was started by adding 15 µl prothrombin in the same buffer. Initial rates of prothrombin activation i.e. thrombin plus meizothrombin formation (panel A-C) and of meizothrombin formation were determined as described under Experimental Procedures. Meizothrombin formation (Panel D-F) is expressed as the percentage of the total amount of enzymatically active prothrombin activation product (meizothrombin plus thrombin) formed. The final reaction conditions were:
Panel A,D: 50 mM of a mixture of Mes, Hepes and Tris to obtain the desired pH at 37 °C, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 2 mM $CaCl_2$, 4 pM Xa, 5 nM Va, 100 µM DOPS/DOPC vesicles (20/80; mole/mole) and 2 µM prothrombin.
Panel B,E: 50 mM Hepes (pH 7.5 at 37 °C), NaCl as indicated in the figure and further concentrations of reactants as under A,D.
Panel C,F: 50 mM Hepes (pH 7.5) and further concentrations of reactants as under A,D. For each temperature the pH of the reaction buffer was adjusted to pH 7.5.

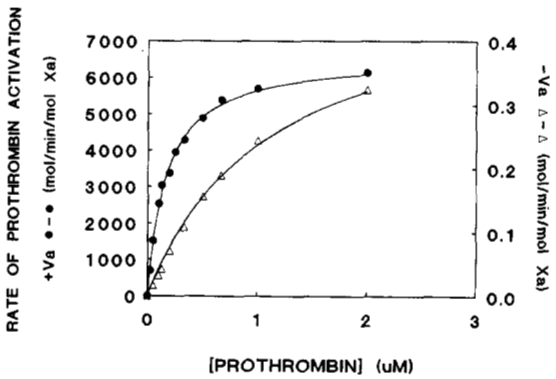

Figure 2A. Initial rates of prothrombin activation as a function of prothrombin concentration.
The initial rate of prothrombin activation i.e. thrombin plus meizothrombin formation and of meizothrombin formation were determined at varying prothrombin concentrations in the absence or presence of factor Va as described under Experimental Procedures. Initial rates of prothrombin activation are given here (Fig. 2A) whereas meizothrombin formation, expressed as percentage of the total amount of enzymatically active prothrombin activation product (thrombin plus meizothrombin) formed at each prothrombin concentration is given in Fig. 2B in the results section. Further details are given in the legend to Fig. 1 and in the Experimental Procedures. The final concentrations of reactants were: 50 mM Hepes (pH 7.5 at 37 °C), 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 2 mM $CaCl_2$, 50 µM DOPS/DOPC vesicles (20/80; mole/mole), prothrombin concentrations as indicated in the figure and 4 nM factor Xa without factor Va (△-△) or 3 pM factor Xa and 5 nM factor Va (●-●).

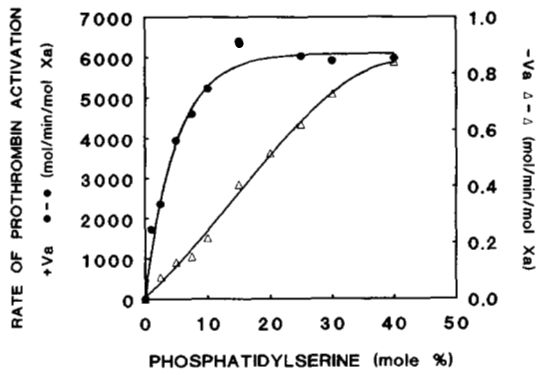

Figure 3A. Effect of the membrane composition on prothrombin activation.
The initial rate of prothrombin activation and of meizothrombin formation were determined on DOPS/DOPC vesicles of varying composition both in the absence and presence of factor Va. The dependence of the initial rate of prothrombin activation on membrane composition is given here (Fig. 3A) whereas meizothrombin formation, expressed as percentage of the total amount of enzymatically active prothrombin activation product (thrombin plus meizothrombin) formed at each phospholipid composition is given in Fig. 3B of the results section. Further details are given in the legend to Fig. 1 and in the Experimental Procedures. The final concentrations of reactants were: 50 mM Hepes (pH 7.5 at 37 °C), 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 50 µM DOPS/DOPC vesicles with varying mole% DOPS as indicated in the figure, 2 µM prothrombin, 2 mM $CaCl_2$ and 3.5 nM factor Xa and no factor Va (△-△) or 3.5 pM factor Xa and 5 nM factor Va (●-●).

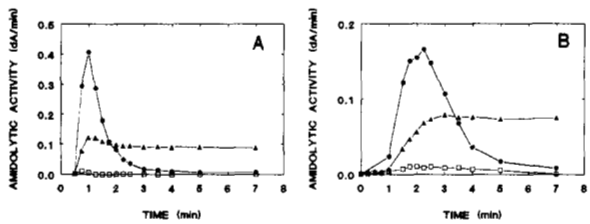

Figure 7. Time course of prothrombin activation during activation of plasma with kaolin in the presence of cephalin (A) and in the presence of collagen stimulated platelets (B).
A. 400 µl Defibrinated normal human plasma was preincubated at 37 °C with 50 µl buffer for 5 min after which 50 µl of a kaolin/cephalin/$CaCl_2$ mixture was added to start activation. Final concentrations reached were: 0.8 volume platelet poor plasma, 25 µM cephalin (based on phosphate analysis), 1 mg/ml kaolin, 20 mM Hepes (pH 7.5 at 37 °C) and 13 mM added $CaCl_2$. At the time points indicated 10 µl aliquots were withdrawn and assayed for prothrombin activation products as described in the Experimental Procedures. Symbols represent the amidolytic activity (arbitrary units) of: ●-●, thrombin; □-□, meizothrombin plus meizothrombin-des-fragment 1; ▲-▲, α_2-macroglobulin inhibited products. The highest value of thrombin reached equalled 0.41 µM.
B. To 350 µl defibrinated normal human plasma 100 µl washed human platelets ($1x10^9$/ml) were added and the mixture was incubated at 37 °C. After 5 min activation was started with the addition of 50 µl of a kaolin/collagen mixture. The reaction was carried out under stirring conditions to ensure optimal expression of platelet procoagulant activity (35). Final concentrations reached were: 0.7 volume platelet poor plasma, 2x108/ml platelets, 10 µg/ml collagen, 1 mg/ml kaolin, 20 mM Hepes (pH 7.5 at 37 °C) and 13 mM added $CaCl_2$. At the time points indicated 10 µl aliquots were withdrawn and assayed for prothrombin activation products as described in the Experimental Procedures. Symbols represent the amidolytic activity (in arbitrary units) of: ●-●, thrombin; □-□, meizothrombin plus meizothrombin-des-fragment 1; ▲-▲, α_2-macroglobulin inhibited products. The highest value of thrombin reached equalled 0.17 µM.

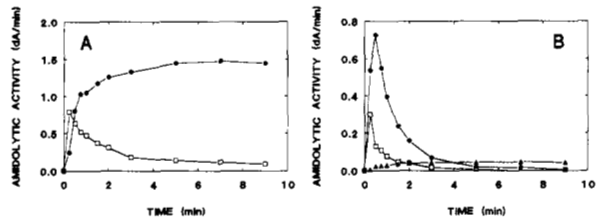

Figure 8. Time course of prothrombin activation in the absence and in the presence of antithrombin III and α_2-macroglobulin.
Prothrombin (1.5 µM) was activated in a total volume of 300 µl containing 10 mM Hepes, 136 mM NaCl, 2.68 mM KCl, 2 mM $MgCl_2$, 25 mM glucose, 5 mg/ml BSA, 0.5 nM factor Xa, 5 nM factor Va, 2 mM $CaCl_2$ and 100 µM DOPS/DOPC vesicles (20/80:mole/mole) in the absence (Panel A) and in the presence of 1.6 µM antithrombin III and 2.5 µM α_2-macroglobulin (Panel B). At the time poins indicated aliquots were withdrawn from the reaction mixture and assayed for prothrombin activation products as described in the legend to Fig. 6 and under Experimental Procedures. Symbols represent the amidolytic activity (in arbitrary units) of: ●-●, thrombin; □-□, meizothrombin plus meizo–thrombin-des-fragment 1; ▲-▲, α_2-macroglobulin-inhibited products. The highest values of thrombin and meizothrombin reached were 1.47 µM and 0.8 µM (Panel A) and 0.73 and 0.3 µM (Panel B).